

**Anti-remodeling agents influence osteoblast activity differently in modeling- and remodeling-sites of canine rib**

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## 1 ABSTRACT

2 Anti-remodeling agents reduce bone loss in part through direct actions on osteoclasts.  
3 Their effects on osteoblasts and bone formation activity are less clear and may differ at  
4 sites undergoing modeling versus remodeling. Skeletally-mature intact beagles, which  
5 were 1-2 years old at the start of the study, were treated daily with clinically relevant  
6 doses alendronate (0.10 or 0.20 mg/kg/day), risedronate (0.05 or 0.10 mg/kg/day),  
7 raloxifene (0.50 mg/kg/day), or vehicle (1 ml/kg/day). Dynamic bone formation  
8 parameters were histologically assessed on periosteal, endocortical/trabecular, and  
9 intracortical bone envelopes of the rib. Raloxifene significantly increased periosteal  
10 surface mineral apposition rate (MAR), a measure of osteoblast activity compared to all  
11 other treatments (+108 to +175%;  $p < 0.02$ ) while having no significant effect on MAR at  
12 either the endocortical/trabecular or intracortical envelopes. Alendronate (both 0.10 and  
13 0.20 doses) and risedronate (only the 0.10 dose) significantly ( $p \leq 0.05$ ) suppressed MAR  
14 on the endocortical/trabecular envelope, while none of the bisphosphonate doses  
15 significantly altered MAR at either the periosteal or intracortical envelopes compared to  
16 vehicle. Based on these results we conclude 1) at clinically-relevant doses the two  
17 classes of anti-remodeling agents, bisphosphonates and SERMs, exert differential effects  
18 on osteoblast activity in the canine rib and 2) this effect depends on whether modeling or  
19 remodeling is the predominant mechanism of bone formation.

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23 **Key words:** Bisphosphonates, raloxifene, alendronate, risedronate, periosteal

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**1 INTRODUCTION**

2           Anti-remodeling agents significantly reduce vertebral fracture risk in part through  
3 their direct effects on osteoclasts. By inducing osteoclast apoptosis and by suppressing  
4 activity/proliferation/differentiation [1-5], the number of newly initiated remodeling sites  
5 is reduced [6]. Due to the coupling of resorption and formation activity during  
6 remodeling, anti-remodeling agents effect a systemic decrease in bone formation activity  
7 [7, 8]. It is unclear whether these agents have separate effects on osteoblasts independent  
8 of their suppression of remodeling.

9           Numerous studies have attempted to determine the effects of anti-remodeling  
10 agents on osteoblasts. In vitro data show positive effects of bisphosphonates on  
11 osteoblast proliferation and differentiation [9-11], as well as suppression of apoptosis [12,  
12 13]. These data are contrasted by in vivo animal studies that have shown bisphosphonate  
13 suppression of mineral apposition rate, indicative of reduced osteoblast activity, at both  
14 modeling and remodeling sites [14-17]. Selective estrogen receptor modulators (SERMs)  
15 suppress osteoblast apoptosis [18] and increase osteoblast differentiation [19] and  
16 proliferation [20] in vitro. In vivo, SERMs have produced conflicting results with respect  
17 to effects on mineral apposition rate [21-23].

18           Anti-remodeling agents could exert differential effects on osteoblasts depending  
19 on whether the cells are involved in remodeling or modeling bone formation activity.  
20 Remodeling-associated formation activity, the predominant form on endocortical,  
21 trabecular, and intracortical envelopes [24, 25], is coupled to osteoclasts. Anti-  
22 remodeling agents may indirectly reduce the individual activity of osteoblasts through  
23 reductions in BMU-level osteoclast activity (i.e. reduced erosion depth) [26]. These

agents may also directly influence osteoblasts in vivo. This effect would be most evident at sites undergoing modeling, where formation occurs without previous resorption. Modeling is the predominant mechanism of formation activity on periosteal surfaces although it can occur on other surfaces [24, 25].

The goal of this study was to determine the effect of bisphosphonates (alendronate and risedronate) and a SERM (raloxifene) on mineral apposition rate, an indicator of osteoblast activity. Following one-year treatment of intact beagle dogs with clinically relevant doses of these agents, ribs were histologically assessed for bone formation variables. Mineral apposition rate was used as an indicator of osteoblast activity and was assessed separately at sites that predominately undergo remodeling (endocortical/trabecular and intracortical envelopes) or modeling (periosteal) bone formation. We hypothesized that these three anti-remodeling agents would all suppress osteoblast activity (mineral apposition rate) at both modeling and remodeling sites.

## **MATERIALS AND METHODS**

### **Animals**

Seventy-two skeletally mature female beagles ( $1.3 \pm 0.02$  years old) were purchased from Marshall Farms USA (North Rose, NY). Upon arrival, lateral X-rays of all dogs were obtained to confirm skeletal maturity (closed proximal tibia and lumbar vertebra growth plates). Animals were housed two per cage in environmentally controlled rooms at Indiana University School of Medicine's AALAC accredited facility and provided standard dog chow and water. All procedures were approved prior to the study by the Indiana University School of Medicine Animal Care and Use Committee.

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**Experimental Design**

Following two weeks of acclimatization, animals were assigned to treatment groups (n=12/group) by matching body weights. All dogs were treated daily for 1-year with oral doses of vehicle (saline, 1.0 ml/kg/day), risedronate sodium (0.05 or 0.10 mg/kg/day; Procter and Gamble Pharmaceuticals, Inc), alendronate sodium (0.10 or 0.20 mg/kg/day; Merck and Co., Inc), or raloxifene (0.50 mg/kg/day; Lilly Research Labs, Indianapolis, IN). The bisphosphonate doses (RIS 0.10 and ALN 0.20) are equivalent to those used for treatment of post-menopausal osteoporosis on an mg/kg basis, the lower doses of each drug correspond to approximately ½ the clinical treatment dose. The raloxifene dose was chosen to produce serum levels approximately equivalent to those documented in post-menopausal women. Risedronate and alendronate were dissolved in saline; raloxifene was diluted in 10% hydroxypropyl-β-cyclodextrin made with distilled water. All agents were administered orally with a syringe each morning after an overnight fast and at least two hours prior to feeding. Prior to necropsy, animals were injected with calcein (0.20 mL/kg, IV) using a 2-12-2-5 labeling schedule (9 animals per group) or a 2-5-2-5 (3 animals per group). The shorter interlabel duration was due to a scheduling error. Animals were euthanized after 1 year by intravenous administration of sodium pentobarbital (0.22 mg/kg Beuthanasia-D Special). After death, the right ninth rib (~20 mm) was dissected, placed in 10% neutral buffered formalin for 72 hours, and then transferred to 70% ethanol for processing.

**Histology**

Using an automatic tissue processor (Shandon/Lipshaw), specimens were cycled through a graded series of ethanols, cleared using xylene, and infiltrated with methyl methacrylate (MMA; Aldrich) using routine embedding procedures. Transverse sections (80-100  $\mu\text{m}$ ) were cut using a diamond wire saw (Histosaw; Delaware Diamond Knives). Histological measurements were made using a semiautomatic analysis system (Bioquant OSTEO 7.20.10, Bioquant Image Analysis Co.) attached to a microscope equipped with an ultraviolet light source (Nikon Optiphot 2 microscope, Nikon). Measurements were made on one cross-section per animal (**Figure 1**). Periosteal, endocortical/trabecular (MS/BS, MAR, BFR/BS), and intracortical (MAR, Labeled osteon number, Ac.f) dynamic bone formation parameters were separately analyzed as previously described [16] and conformed to standard ASBMR nomenclature [27]. Endocortical and trabecular surfaces were analyzed as a single entity as it was not possible to consistently define the boundary between two surfaces across all tissue sections.

## Statistics

Statistical tests were performed using SAS software (SAS Institute, Inc.). Differences among treatment groups were evaluated using a one-way analysis of variance (ANOVA). When a significant overall F value ( $p \leq 0.05$ ) was present, differences between individual group means were tested using Fisher's protected least-significant difference (PLSD) post-hoc test. For all tests,  $p \leq 0.05$  was considered statistically significant. Data are presented as mean  $\pm$  standard error.

## RESULTS

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1 The two classes of drugs, bisphosphonates (alendronate and risedronate) and SERMs  
2 (raloxifene), exerted envelope-specific effects on mineral apposition rate (MAR) of non-  
3 ovariectomized dog ribs (Table 1, Figure 2). Raloxifene-treated animals had significantly  
4 higher MAR (+108%;  $p = 0.019$ ) on the periosteal surface compared to animals treated  
5 with vehicle (Figure 2). Neither alendronate nor risedronate significantly altered  
6 periosteal MAR at either treatment dose compared to vehicle-treated animals. Periosteal  
7 surface MAR was significantly higher in raloxifene-treated animals compared to all  
8 bisphosphonate-treated groups (+112 to + 175%; all  $p < 0.05$ ). Endocortical/trabecular  
9 MAR was similar between vehicle-and raloxifene-treated animals yet was significantly  
10 higher in raloxifene-treated animals compared to all bisphosphonate-treated groups (+ 53  
11 to + 464%; all  $p < 0.05$ ) (Figure 2). MAR was significantly lower than vehicle in animals  
12 treated with alendronate at the 0.10 dose (-59%) and the 0.20 dose (-41%), as well as  
13 risedronate at the 0.10 dose (-80%). There was no difference in MAR between vehicle  
14 and the lower dose of risedronate (0.05) on the endocortical/trabecular surface.  
15 Intracortical MAR was not altered by any of the treatments.

16 Periosteal mineralizing surface (MS/BS) was not significantly different ( $p = 0.22$ )  
17 among the treatment groups (Table 1). On the endocortical/trabecular surface, there was  
18 no significant effect of raloxifene on MS/BS compared to vehicle. However, raloxifene-  
19 treated animals had significantly higher MS/BS on this surface compared to all  
20 bisphosphonate-treated groups (+60 to + 254%; all  $p < 0.05$ ). Endocortical/trabecular  
21 MS/BS was significantly lower than vehicle in groups treated with alendronate (both 0.10  
22 and 0.20 doses) or risedronate (only the 0.10 dose). Labeled osteon number, the

1 surrogate to MS/BS within the intracortical envelope, was not significantly altered by any  
2 of the treatments (Table 1).

3 Periosteal surface bone formation rate was significantly higher in raloxifene-  
4 treated animals compared to both vehicle (+285%;  $p < 0.003$ ) and all bisphosphonate-  
5 treated groups (Table 1). There was no significant difference between vehicle and any of  
6 the bisphosphonate-treated groups. Endocortical/trabecular surface BFR was unchanged  
7 with raloxifene compared to vehicle; bisphosphonate-treated animals (ALN 0.10 and  
8 0.20; RIS 0.10) had significantly lower BFR compared to vehicle. Intracortical activation  
9 frequency was not different among the groups.

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## 11 **DISCUSSION**

12 In addition to direct effects on osteoclasts, anti-remodeling agents may also influence  
13 osteoblasts. It is well accepted that these agents reduce bone formation but whether this  
14 is due to a decrease in the number of formation sites, a decrease in formation at each  
15 individual site, or both, is unclear. In vitro studies have shown that both bisphosphonates  
16 and SERMs have direct positive effects on osteoblasts [9-13, 18-20]; however in vivo  
17 data have produced conflicting results. Our data show that following one-year treatment  
18 of intact dogs with clinically relevant doses, raloxifene significantly stimulates osteoblast  
19 activity on periosteal surfaces without an effect on endocortical/trabecular surfaces. In  
20 contrast to raloxifene, bisphosphonates do not alter periosteal osteoblast activity while  
21 significantly suppressing mineral apposition rate on endocortical/trabecular surfaces.

22 In the mature skeleton, periosteal bone formation occurs predominately through  
23 formation without prior resorption [24]. Our data show raloxifene significantly increases



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osteoblast activity (assessed by MAR) on the rib periosteal surface compared to vehicle-treated animals. This is consistent with in vitro data showing raloxifene can positively affect osteoblast activity. In culture, raloxifene stimulates type 1 collagen secretion and alkaline phosphatase activity [19, 20], and suppresses osteoblast apoptosis [18]. In our study there was no significant effect of raloxifene (positive or negative) on mineral apposition rate within either the endocortical/trabecular or intracortical envelopes of the rib, both sites that predominately undergo coupled formation and resorption in the adult skeleton. These data suggest raloxifene has a direct stimulatory effect on osteoblasts associated with formation on a surface that primarily undergoes modeling while this effect is negated on surfaces that primarily undergo remodeling-associated formation activity.

The mechanism through which raloxifene stimulates osteoblast activity on the periosteal surface is unclear. The estrogen receptor-alpha (ER $\alpha$ ) is more highly expressed in cortical bone [28] and appears to be a major regulator of bone modeling on the periosteal surface. Mice lacking ER $\alpha$  receptors exhibit reduced periosteal diameter [29, 30] and an attenuation of loading-induced periosteal formation [31]. Animals and cells lacking ER $\beta$  are minimally affected with respect to periosteal geometry or cellular activity [32]; this receptor appears to play a greater role in endocortical and trabecular sites. Our findings may be explained by the selective modulation of ER $\alpha$  or ER $\beta$  by raloxifene which may differentially regulate osteoblast related genes [33] at the various bone envelopes depending on the estrogen receptor population expressed. It is also possible that factors associated with osteoclasts and/or resorption offset the positive effect of raloxifene at remodeling sites.

1 Periosteal expansion significantly increases bone strength [34]. The relationship  
2 between periosteal dimensions and bone strength is exponential; increases of periosteal  
3 radii enhance section modulus (an estimator of bone strength) by the fourth power [35].  
4 Thus, small increases in periosteal apposition are mechanically advantageous as limited  
5 amounts of new bone can substantially increase fracture resistance and can mechanically  
6 offset loss of endocortical/trabecular bone. The increases in MAR (and BFR) with  
7 raloxifene would be expected to significantly increase non-vertebral bone cross sectional  
8 area and reduce non-vertebral fracture risk, yet neither of these effects has been  
9 confirmed in clinical trials. Raloxifene did not enhance periosteal expansion beyond that  
10 of placebo-treated post-menopausal women after 3 years [36] although the technique  
11 used to assess bone size (DXA images) may not have sufficient resolution to detect small  
12 differences. Additionally, clinical trials with raloxifene have not shown a significant  
13 reduction in non-vertebral fractures [37] (in contrast to both risedronate [38] and  
14 alendronate [39]). Increased trabecular BMD with bisphosphonates is largely responsible  
15 for the reduced fracture risk. Because raloxifene is a less potent anti-remodeling agent it  
16 has less effect on trabecular BMD which may explain its failure to significantly reduce  
17 non-vertebral fracture risk. Still, it is entirely possible that periosteal expansion, although  
18 known to increase bone strength, is not sufficient with raloxifene-treatment to decrease  
19 fracture risk in the absence of a more significant trabecular BMD response.

20 Bisphosphonate treatment (either alendronate or risedronate) did not significantly  
21 alter periosteal surface MAR but did significantly suppressed MAR on  
22 endocortical/trabecular surfaces. These effects on different bone surfaces are consistent  
23 with previous studies in dogs [16, 40] [17, 41] and nonhuman primates [42]. In growing

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[14] and skeletally mature [15] rats, however, treatment with either alendronate or risedronate significantly suppresses osteoblast activity on both periosteal and endocortical surfaces. Our data suggest that bisphosphonates negatively influence the work rate of osteoblasts associated with remodeling-associated formation. High concentrations of bisphosphonates are known to be liberated from the bone matrix during resorption, and osteoblasts have been shown to internalize bisphosphonates [43]. Since bisphosphonates cannot be metabolized, sufficient uptake in osteoblasts would compromise their function via inhibition of protein prenylation [44]. We therefore hypothesize that decreases in formation with bisphosphonates is due to both a decrease in the number of forming sites and the osteoblast activity at each site. However, human data quantifying MAR with bisphosphonate treatment generally show no effect at any bone envelope [6, 26, 45, 46]. Although these human data are limited by relatively small sample sizes and are confined to the iliac crest, until similar drug effects on osteoblast activity can be confirmed in humans our interpretations remain a hypothesis.

Periosteal bone formation can be a compensatory mechanism to maintain bone strength in situations where bone loss occurs from trabecular and endocortical surfaces [47]. At their respective clinical doses, raloxifene is known to suppress remodeling less than the bisphosphonates [48]. In these same dogs, vertebral bone turnover was suppressed ~70% with ALN 0.2 and RIS 0.1, and only ~20% with RAL, compared to vehicle [49]. It is therefore possible that bisphosphonates did not increase periosteal formation in our study because the suppression of remodeling on endocortical/trabecular surfaces was sufficient to increase bone mass to some critical level. If this were the case, one might expect that a bisphosphonate dose that had less suppressive effect on the

1 endocortical/trabecular surface would have a more significant anabolic effect on the  
2 periosteal surface. However, we did not find this to be the case, as the lower dose of  
3 risedronate (0.05) suppressed endocortical/trabecular bone formation significantly less  
4 than the higher dose of risedronate (0.10), yet the two had similar periosteal bone  
5 formation parameters. These data suggest that the level of turnover suppression on  
6 endocortical/trabecular surface is not a main determinant of periosteal osteoblast activity.

7 Our data describing bisphosphonate suppression of MAR at remodeling sites may  
8 provide some explanation for results from clinical trials using combination treatments.  
9 Treatment with alendronate, either concurrently or sequentially with teriparatide, blunts  
10 teriparatide-induced increases in bone formation biomarkers and BMD of post-  
11 menopausal women [50, 51] and men with low bone mass [52]. This blunting was  
12 hypothesized to be related to the reduction in the number of remodeling sites by  
13 alendronate, leaving few active formation sites for teriparatide to stimulate. Based on our  
14 data, an alternative hypothesis may be that bisphosphonate suppression of osteoblast  
15 activity directly offsets the osteoblast stimulatory effect of teriparatide. Additionally, a  
16 suppressive effect of bisphosphonates on remodeling-associated bone formation activity  
17 could help explain the failure to consistently find increased trabecular bone volume in  
18 bisphosphonate clinical trials [6, 53].

19 One aim of this study was to compare the effects of raloxifene and  
20 bisphosphonates at doses corresponding to those used for treatment of post-menopausal  
21 women. Post-study analyses of serum from three raloxifene-treated dogs, for reasons  
22 unrelated to this study, revealed that the serum concentration of raloxifene was  
23 approximately  $\frac{1}{2}$  that predicted from the original dosing calculations. These serum levels

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were still within the range quantified in post-menopausal women receiving the 60 mg/day dose of raloxifene (Lilly data on file). We therefore have compared the raloxifene dose to both the clinical and ½ clinical dose of each bisphosphonate. Based on the similarity among the four different bisphosphonate-treated groups with respect to periosteal MAR, the significantly greater MAR with raloxifene appears to represent a true difference in the biological activity of the drug administered at clinically-relevant doses. We cannot discount the possibility, however, that bisphosphonate doses lower than those used in this study could potentially stimulate periosteal formation to levels comparable with raloxifene.

The current study has various limitations that should be noted. We analyzed only one bone site of intact, non-ovariectomized beagle dogs and therefore cannot be certain whether similar changes would occur in the absence of estrogen (i.e. post-menopausal women), or at other bone sites. The endocortical/trabecular surfaces were not separately assessed as the boundaries are difficult to differentiate and in our experience the surfaces respond in similar ways. Although raloxifene dosing levels provided serum values within the range quantified in post-menopausal women receiving the 60 mg/day dose of raloxifene (Lilly data on file) we cannot discount the possibility that administering the drug at a higher mg/kg dose would produce different results. Although there is no data to suggest the raloxifene carrier has any effect on bone formation (10% hydroxypropyl-β-cyclodextrin), we cannot exclude this possibility. Finally, although all growth plates are generally closed in the dog by 12 months [54], we did not assess the rib growth plates prior to treatment and therefore do not know whether continued growth occurred in the ribs of these animals during the course of the study.

1 In conclusion, we have shown that one year of treatment with clinically relevant  
2 doses of anti-remodeling agents significantly influences osteoblast activity in the rib of  
3 beagle dogs. Raloxifene stimulates modeling-associated osteoblast activity on the  
4 periosteal surface while maintaining remodeling-associated activity on  
5 endocortical/trabecular surfaces at levels similar to untreated controls. In contrast,  
6 bisphosphonates suppress remodeling-associated osteoblast activity on the  
7 endocortical/trabecular surface while having no significant effect on periosteal osteoblast  
8 activity.

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9     Health.

For Peer Review

## Figure Legends

**Figure 1.** Photomicrograph of rib cross-section. Calcein labeling was separately quantified on periosteal (P), endocortical/trabecular (E/T), and intracortical (I) bone envelopes. Scale bar = 1mm.

**Figure 2.** Envelope-specific effects of anti-remodeling agents on mineral apposition rate (MAR) of dog ribs following 1-year treatment with clinically relevant doses of raloxifene (RAL), alendronate (ALN), or risedronate (RIS). (A) Periosteal MAR; (B) endocortical/trabecular envelope MAR. Data presented as mean  $\pm$  SE.  $p < 0.05$  vs <sup>a</sup> all other treatments, <sup>b</sup> all bisphosphonate-treated groups, <sup>c</sup> VEH, <sup>d</sup> RIS 0.05, <sup>e</sup> RIS 0.1.



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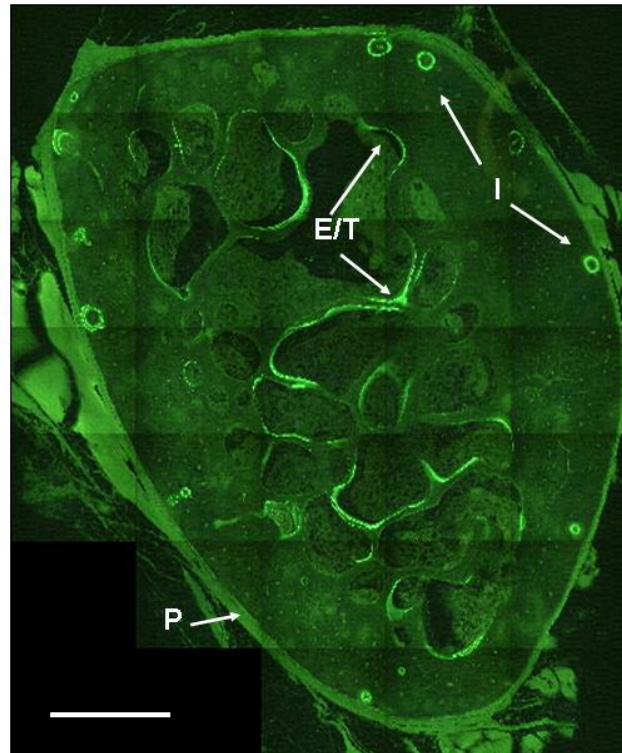
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**Table 1.** Rib bone formation parameters on periosteal, endocortical/trabecular, and intracortical envelopes.

	Vehicle	Raloxifene	Risedronate		Alendronate		P value
	1 ml/kg/d	0.5 mg/kg/d	0.05 mg/kg/d	0.1 mg/kg/d	0.1 mg/kg/d	0.2 mg/kg/d	
<b>Periosteal</b>							
MS/BS, %	7.7 ± 1.5	17.6 ± 2.5	14.0 ± 2.3	13.6 ± 3.9	16.1 ± 2.7	13.8 ± 2.8	0.22
BFR, um <sup>3</sup> /um <sup>2</sup> /day	5.4 ± 1.8	20.8 ± 4.1 <sup>a</sup>	8.0 ± 3.2	9.6 ± 3.5	11.1 ± 3.2	9.4 ± 3.6	0.05
<b>Endocortical/Trabecular</b>							
MS/BS, %	22.1 ± 3.3	26.6 ± 3.2 <sup>b</sup>	16.6 ± 3.5	9.4 ± 2.3 <sup>c</sup>	9.0 ± 2.4 <sup>c</sup>	7.5 ± 1.6 <sup>c</sup>	< 0.0001
BFR, um <sup>3</sup> /um <sup>2</sup> /day	28.7 ± 4.8	36.7 ± 4.9 <sup>b</sup>	18.6 ± 5.5	4.7 ± 2.7 <sup>cd</sup>	6.0 ± 2.6 <sup>cd</sup>	6.5 ± 1.6 <sup>c</sup>	< 0.0001
<b>Intracortical</b>							
MAR, um/day	0.93 ± 0.14	0.98 ± 0.14	1.07 ± 0.09	0.97 ± 0.14	1.02 ± 0.06	0.89 ± 0.13	0.89
L.On.N, #/mm <sup>2</sup>	1.43 ± 0.21	1.80 ± 0.42	1.88 ± 0.27	1.45 ± 0.31	2.10 ± 0.49	1.59 ± 0.45	0.78
Ac.f, #/year	13.9 ± 3.1	14.8 ± 3.1	15.1 ± 2.9	13.0 ± 3.3	17.0 ± 4.2	12.9 ± 3.5	0.96

Data are mean ± SE of n=12 per group. p < 0.05 vs <sup>a</sup>all other treatments, <sup>b</sup>all bisphosphonate-treated groups, <sup>c</sup>VEH, <sup>d</sup>RIS 0.05, <sup>e</sup>RIS 0.1. MAR, mineral apposition rate; MS/BS, mineralizing surface per bone surface; BFR, bone formation rate; L.On.N, labeled osteon number; Ac.f., activation frequency.

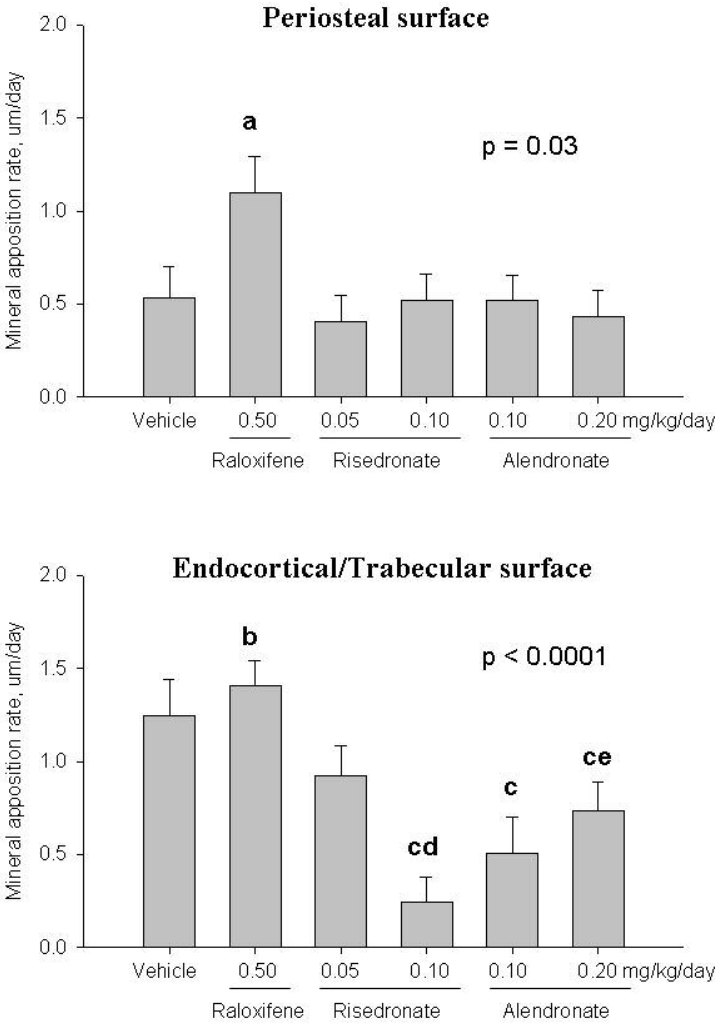
Figure 1



**Rib image showing regions of histological analyses. Separate measures were made on calcein labeled surfaces of periosteal (P), endocortical/trabecular (E/T), and intracortical (I) bone envelopes. Scale bar = 1mm.**



Figure 2



**Figure 2. Envelope-specific effects of anti-remodeling agents on mineral apposition rate (MAR) of dog ribs following 1-year treatment with clinically relevant doses of raloxifene (RAL), alendronate (ALN), or risedronate (RIS). (A) Periosteal MAR; (B) endocortical/trabecular envelope MAR. Data presented as mean  $\pm$  SE.  $p < 0.05$  vs a all other treatments, b all bisphosphonate-treated groups, c VEH, d RIS 0.05, e RIS 0.1.**